Appendices

Appendix 1 - Chapter 5

Calculating the number of transcripts per cell

Single-probe fluorescence *in situ* hybridization (spFISH) technology was employed to quantify the transcripts in single cells. We previously showed that non-induced cells displayed defined fluorescent spots and that this was due to probe hybridization with plasmid DNA undergoing rolling circle replication (Chapter 2). Even after opimization only seven TAMRA molecules on average were detectable after hybridization, which is far less than the maximum theoretical incorporation of twenty-four TAMRA molecules. We therefore concluded that the low and heterogeneous hybridization efficiencies and/or quenching of the TAMRA-labeled probes complicated their distinction from background noise and thereby the possibility to quantify the number of transcripts per detected spot. However, spFISH still enabled us to *roughly* estimate the mRNA numbers per cell using the following protocol. We assumed that the number of probes binding to one plasmid is similar to the number of probes binding to one mRNA molecule, since both molecules contain twelve probe-annealing sites. Therefore, the fluorescence parameters of these spots can be used to estimate the total transcript number per cell. All images were processed as follows: The average fluorescence background signal in cells was determined per pixel, which value was subtracted from each pixel signal on the entire image, preserving only signals above the background signal in cells. A spot detection plug-in was used to collect fluorescent spots in the background-subtracted images of non-induced cells and to fit Gaussian fitting parameters to each spot (ISBatch ImageJ plug-in). The fluorescent volume (V_f) of each spot was calculated according to equation 5.4.

$$V_f = 2\pi A \sigma_x \sigma_y \qquad \textit{(equation 5.4)}$$

A is the amplitude of the fitted 2d Gaussian function, whereas σ_x and σ_y are the width parameters in the x and y direction, respectively. A total of 500 spots were used to determine the frequency distribution of all measured V_f values and to estimate the V_f corresponding to one mRNA molecule (**Fig 1**). However, since probe-annealing efficiency is never 100%, we assumed that the V_f value at the highest frequency is likely an underestimation. We fitted a normal distribution on the acquired data assuming that most of the MS2 binding

sites on the replicating plasmids are not fully occupied (**Fig 1**). The number on the top end (4000 AU) was therefore used for further analysis. We then measured the total fluorescence of each cell in the background-subtracted images using ImageJ, with cell meshes obtained using the Oufti software. The sum of the values of the pixels in each mesh, divided by the V_f value of one mRNA molecule, was used to get a rough estimation of transcript quantity per cell. To account for the presence of plasmids that undergo replication, each transcript number was subtracted with two (the average number of replicating plasmids per cell in non-induced cells) . This process was repeated for images of cells induced with a gradient of nisin concentrations, after which the median transcript number of all the cells and of only induced cells in the captured population was determined.

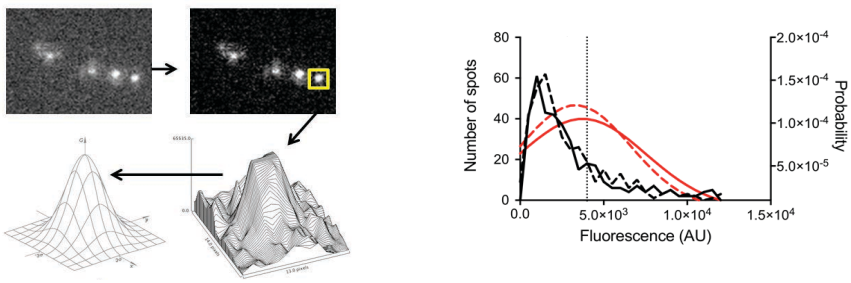


Fig 1. Flow of data handling for the determination of transcript numbers. Left panels: Determination of transcript numbers per cell using the following steps (from top left left to bottom left): background subtraction, spot detection, gaussian parameter fitting. See the text for an in-depth description of each step. Right panel: determination of the frequency distribution (right *y*-axis, red lines) of all measured V_f values (left *y*-axis, black lines) of two separate data sets and the estimation of the V_f value corresponding to one mRNA molecule (grey dotted line = 4000 AU).

Appendix 2 - Chapter 5

Modeling mRNA and protein expression of the NICE system

A two-state model of mRNA and protein production was employed as illustrated in **Fig 1A**. The model describing the NICE expression parameters over time simulated in the MATLAB-based SimBiology toolbox, using the parameters depicted in **Fig 1B**. The production rate ($k1$) of $bcaP_{12bs}$ and $PS1\Delta9_{12bs}$ mRNA production was introduced as a function of the concentration of nisin used to induce the system. The relationship between nisin concentration and $k1$ is given in **Fig 7D of Chapter 5** and can be best described by a cubic polynomial function (equation 5.5), where i is the level of nisin added to the system.

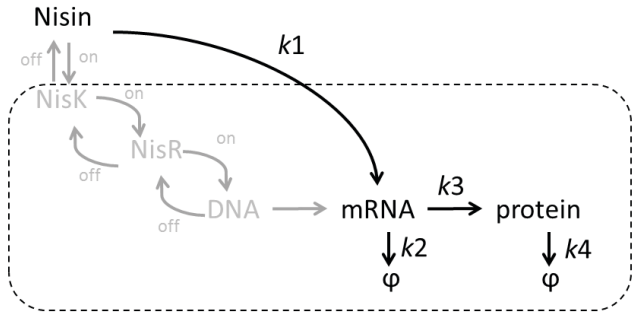
$$k1(i) = A_1 i^2 + A_2 i + A_3 \qquad (equation\ 5.5)$$

To simulate the effect of cell growth reaching stationary phase, the values for i, $A3$ and $k4$ were set to 0 at time point 4 (4 hrs after induction with nisin). To mimic the effect of rapid PS1Δ9 accumulation, a threshold was incorporated when protein levels reached a value of 2000 molecules. Two scenarios were incorporated: 1, this threshold leads to a halt in transcription and translation, but cell growth continues; 2, cell division and translation are blocked, but transcription continues.

The effect of expressing transcripts from the *pseudo10* locus instead of from plasmid pNZ8048 was calculated as follows. The production of GFP-tagged proteins from a *gfp* gene either inserted in *pseudo10* (single copy) or pNZ8048 (multiple copies) was previously compared (Chapter 2). A 10-fold reduction in GFP levels in case of expression from the chromosomal *pseudo10* locus was observed. This was accounted for in the model by incorporating a 10-fold reduction of the 'A'-values to obtain the values of $k1_{bcaP}$.

To estimate the minimum number of proteins per cell required for successful production, we assumed an ideal final yield of 1 mg of protein per ml of culture. For a 50-kD protein, this is similar to ~1.2×10^{13} proteins per ml. For a bacterial cell culture with an optical density at 600 nm of 2 (corresponding to ~1.6×10^{9} cells ml^{-1}), this would mean that roughly 7500 protein molecules have to be present per cell.

A



B

	Description	Parameters	BcaP	PS1Δ9	*pse10*	Ref
*k*1	mRNA synthesis ($A1i^2 + A2i + A3$)	*A1*	-1.91 h^{-1}	0.42 h^{-1}	-0.19 h^{-1}	This work
		A2	124.15 h^{-1}	56.63 h^{-1}	12.41 h^{-1}	
		A3	199.8 h^{-1}	102 h^{-1}	19.98 h^{-1}	
*k*2	mRNA degradation		6.6 $mRNA^{-1}$ h^{-1}	3.8 $mRNA^{-1}$ h^{-1}		Chapter 4
*k*3	Protein synthesis		60 $mRNA^{-1}$ h^{-1}	60 $mRNA^{-1}$ h^{-1}		Bionumbers
*k*4	Protein dilution		0.6 h^{-1}	0.6 h^{-1}		This work

Fig 1. Modeling of mRNA and protein expression parameters of the NICE system. (**A**) Schematic representation of NisRK signal transduction and gene expression in a *L. lactis* NZ9000 cell following the addition of nisin. Indicated in black are parameters and rates incorporated in the model. Note that the parameters and reactions annotated in grey are 'a black box' and therefore considered as one single step, represented by *k*1. (**B**) The parameters and their description of the rates, *k*1- *k*4, given in A that were used to model the different scenarios: BcaP or PS1Δ9 expression from pNZ8048 or PS1Δ9 expression from *pseudo10* (*pse10*).